Quorum sensing in *Pseudomonas savastanoi* pv. *savastanoi* and *Erwinia toletana*: role in virulence and interspecies interactions in the olive knot

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ABSTRACT: The olive-knot disease (*Olea europea* L.) is caused by the bacterium *Pseudomonas savastanoi* pv. *savastanoi* (PSV). PSV in the olive-knot undergoes interspecies interactions with the harmless endophyte *Erwina toletana* (ET); PSV and ET co-localize and form a stable community resulting in a more aggressive disease. PSV and ET produce the same type of the *N*-acylhomoserine lactone (AHL) quorum sensing (QS) signal and they share AHLs in planta. In this work we have further studied the AHL QS systems of PSV and ET in order to determine possible molecular mechanism(s) involved in this bacterial inter-species interaction/cooperation. The AHL QS regulons of PSV and ET were determined allowing the identification of several QS-regulated genes. Surprisingly, the PSV QS regulon consisted of only a few loci whereas in ET many putative metabolic genes were regulated by QS among which several involved in carbohydrate metabolism. One of these loci was the aldolase-encoding gene *garL*, which resulted to be essential for both co-localization of PSV and ET cells inside olive knots as well as knot development. This study further highlighted that pathogens can cooperate with commensal members of the plant microbiome.

SIGNIFICANCE OF THIS STUDY: This is a report on studies of the quorum sensing (QS) systems of olive knot pathogen *Pseudomonas savastanoi* pv. *savastanoi* and olive-knot cooperator *Erwinia toletana*. These two bacterial species form a stable community in the olive knot, share QS signals and cooperate resulting in a more aggressive disease. In this work we further studied the QS systems by determining their regulons as well studying QS-regulated genes which might play a role in this cooperation. This represents a unique in vivo interspecies bacterial virulence model and highlights the importance of bacterial interspecies interaction in disease.

INTRODUCTION

The recent dramatic increase of microbiome studies has further evidenced what microbiologists have postulated for many years, that most commonly, microorganisms in nature live as members of complex multispecies communities (1, 2). This has demonstrated that many different microbes live in close proximity to each other; however, aspects of microbe-microbe interactions have thus far been significantly understudied. In addition, multispecies microbial communities existing in association with plants could be influenced by the plant and/or could have consequences on plant health; again very few studies have investigated this likely scenario.

Many bacterial species have been studied for their intraspecies signaling system which is known as quorum sensing (QS) (3). QS involves the production and detection of signal molecules which results in the regulation of gene expression in response to bacterial cell number/density (4). Gram-negative bacteria most commonly use *N*-acylhomoserine lactones (AHLs) as QS signals and in proteobacterial phytopathogens it is involved in the regulation of expression of virulence associated factors in the plant (5-9). An archetypical AHL QS system consists of a LuxI-family AHL synthase and a LuxR-family transcription factor which affects target gene expression upon interaction with the cognate AHL at quorum concentrations (10). AHLs vary in their structure having different acyl chain lengths (from 4 to 20 carbons) and display differences in their oxidation state at position C3. AHL signals can also be involved in interspecies signaling in a community since they are freely diffusible and can thus be detected by different bacterial neighbors. In bacterial pathogenesis, especially in human hosts, it is now

75 becoming recognized that many pathogens interact with other microorganisms which
76 may influence the disease process (11-13).
77 Plant microbial diseases are however still very much considered as being caused by
78 single pure pathogens; nevertheless evidence is also beginning to grow that there can be
79 synergisms between different microorganisms. Recently, a clear example of such
80 synergism has been reported in the olive-knot disease of olive trees (*Olea europea* L.)
81 caused by the bacterium *Pseudomonas savastanoi* pv. *savastanoi* (PSV) (14, 15). PSV
82 possesses a typical LuxI/R AHL QS system and it is involved in virulence since mutants
83 in this system result in significantly smaller knots (15). The bacterial load of the knots
84 (also called tumors) is 50% composed of PSV but also contain a significant proportion of
85 an apparently harmless commensal multispecies bacterial community (16) and some
86 members have been shown to cooperate with PSV resulting in an increase of disease
87 severity (15). More precisely, an *Erwinia toletana* (ET) strain (harmless to the olive
88 plant) isolated from the olive knot increased disease severity (larger olive-knot) when co-
89 inoculated with PSV. In addition, it was demonstrated that ET, *Pantoea agglomerans* and
90 PSV form stable multispecies communities and that they share and communicate via
91 AHLs. Interestingly, ET and PSV synthesize structurally identical AHLs and co-
92 inoculation experiments have evidenced that *E. toletana* can rescue AHL negative
93 mutants of PSV and restore virulence (15). Microscopy studies have also revealed that
94 ET and PSV co-localize in the olive-knot further indicating that the two species are
95 sharing the same niche both benefiting from this stable interaction. In addition, *in silico*
96 recreation of the biochemical metabolic pathways encoded by PSV and ET genomes
97 suggested that metabolic complementarity and/or sharing of metabolites could be

involved in the beneficial interaction established between these two bacterial species (16). In this work we have further studied the AHL QS systems of PSV and ET, both *in vitro* and *in planta*, in order to identify specific molecular determinants involved in this interspecies bacterial interaction. Determination of the PSV and ET QS regulon allowed the identification of several QS-regulated genes putatively involved in numerous metabolic pathways, including the ET aldolase-encoding gene *garL*, which resulted to be essential for both co-localization of PSV and ET cells inside olive knots and full knot development.

RESULTS

The *luxI/R* quorum sensing genes in *Pseudomonas savastanoi* pv. *savastanoi* NCPPB 3335 and *Erwinia toletana* DAPP-PG 735

The olive knot pathogen *Pseudomonas savastanoi* pv. *savastanoi* (PSV) NCPPB 3335 (17, 18) was the first PSV genome sequenced and has been used in several studies of virulence mechanisms (19). This genome harbors a canonical *luxI*/*luxR* pair identical to the previously reported *pssI/R* QS system of PSV DAPP-PG 722 [hereafter named *pssI*/*pssR;* (15)] and two *luxR* solos which do not have a cognate *luxI* partner (Figure 1A). From the primary structure of the two LuxR solos, one likely responds to plant signals (designated as LuxR2) and the other most likely to AHLs (designated as LuxR3) (20, 21). Interestingly, this content of LuxI/R QS elements is conserved in all *P. savastanoi* strains infecting woody plants whose genomes have been sequenced (22-25).

With respect to the olive knot resident and PSV cooperator *E. toletana* (ET), we previously reported that ET DAPP-PG 735 was able to synthesize AHLs via the EtoI/R QS system. The *etoI* mutant, hereafter ETETOI, resulted in no AHL production hence it was concluded that ET possessed one AHL QS system (15). Sequencing of the ET genome (26) and its analysis performed here, surprisingly revealed that ET possessed a second complete canonical AHL QS system. The AHL-responsive transcriptional regulator gene was designated as *tolR* and the autoinducer synthase as *tolI* (Figure 1B).

AHL production by *Pseudomonas savastanoi* pv. *savastanoi* NCPPB 3335 and *Erwinia toletana* DAPP-PG 735

QS and AHL production by PSV NCPPB 3335 has not been addressed so far, thus a *pssI* mutant and its complemented strain, expressing the *pssI* gene from a plasmid, were

132 constructed. PSV NCPPB 3335, the *pssI* mutant and its complemented strain were grown
133 overnight in LB broth and AHLs were extracted from spent supernatants as described in
134 the materials and methods section. C6-AHL production was observed and determined for
135 PSV NCPPB 3335, whereas no AHL production was detected for the Δ*pssI* mutant strain
136 (Table 4 and S1). Interestingly, four types of AHLs (C6-, C8-, 3-oxo-C6- and 3-oxo-C8-
137 AHLs) were identified in the supernatant of the Δ*pssI* complemented strain, indicating
138 that overexpression of *pssI* leads to the production of some types of AHLs not detected in
139 the wild type.
140 We also analyzed AHL production by the EtoI/EtoR and TolI/TolR ET DAPP-PG 735
141 QS systems. Production of six types of AHL was detected for the wild type ET DAPPG-
142 PG 735: 3-oxo-C6-, 3-oxo-C8-, 3-oxo-C10-, C6-, C8- and 3-OH-C6-AHLs (Table 4 and
143 S1). We previously reported that this ET strain produced 3-oxo-C6- and 3-oxo-C8-AHLs
144 [15], thus this analytical chemical analysis revealed a wider spectrum of AHL production.
145 As expected, the ETETOI mutant was unable to produce any type of AHL, while the
146 ETETOI complemented strain restored the biosynthesis of all types of AHLs (Table 4
147 and S1). The ETTOLI showed a defect in the biosynthesis of 3-oxo-C10-AHL and
148 unexpectedly it was not restored via the expression of *tolI* in trans. The summary of the
149 complete AHL analysis in relation to the peak areas of the detected chromatographic
150 peaks are provided in Table S1 and Figure S3.
151 **Transcriptional analysis of quorum sensing genes in PSV NCPPB 3335 and ET**
152 **DAPP-PG 735**
153 We previously observed that a *pssR* mutant of PSV DAPP-PG 722 produced an amount
154 of AHLs similar to the wild type strain, suggesting that the positive feedback loop typical

of AHL QS systems does not occur in PSV. To address this possibility in PSV NCPPB 3335, the *pssI* promoter region was cloned in a promoter probe vector (pMP220) upstream a promoterless *lacZ* gene and β-galactosidase activity was measured in PSV NCPPB 3335 and its derivative *pssI* and *pssR* mutants during their growth. As shown in Figure 2A, the activity of *pssI* promoter was significantly increased in the stationary phase (10 hours incubation) compared to the exponential phase (4 hours incubation) in all three PSV genetic backgrounds. Moreover, no differences in β-galactosidase activity was observed among the three strains in neither log phase nor stationary phase, thus confirming that the typical AHL QS positive feedback loop does not occur in PSV NCPPB 3335.

It was also of interest to study the expression of the ET QS systems; gene promoters of *etoI*, *etoR*, *tolI* and *tolR* were fused to a promoterless *gfp* to perform a comparative *in vitro* transcriptional analysis of both systems in ET, ETETOI and ETTOLI genetic backgrounds. Results showed that *tolI* and *tolR* genes had considerably lower promoters activities in ET compared to the *etoI* and *etoR* promoters (Figure 2B). Additionally, transcription of *etoR* in ETTOLI was enhanced compared to ET and ETETOI, suggesting that the TolI/TolR system might repress *etoR* transcription. Taking into account the low activity of *tolI*/*tolR* promoters under the *in vitro* conditions used, we questioned if this system was activated *in planta*. To examine this possibility, co-inoculation of PSV with ET wild type harboring *tolI* promoter fused to GFP were carried out in micropropagated olive plants. No GFP fluorescence was detected for *tolI* promoter, whereas it was observed in the *etoI* promoter fusion, thus demonstrating that *tolI* gene expression was very low also *in planta*. We then decided to perform a comparative analysis by RT-qPCR

of the transcription of *tolI* and *tolR* genes in two different media: the King's B rich medium and the Hrp-inducing medium which mimics the plant apoplast (27). Results of this experiment revealed a repression of both genes in the Hrp-inducing medium compared to King's B (Figure S1), which suggests that the environment of the plant might repress *tolI* and *tolR* transcription. It was therefore concluded that the TolI/R system was functional however it was repressed and/or not activated in ET under laboratory and *in planta* conditions that we have used. It cannot also be excluded that this AHL QS system is functional at very low AHL concentrations.

Identification of the PSV NCPPB 3335 quorum sensing regulon

It was of interest to establish the loci regulated by the PssI/R system thus a whole-genome transcriptional RNAseq comparative analysis of wild type PSV NCPPB 3335 and its derivative *pssI* mutant was performed. RNA was extracted from these strains grown in biological triplicates in LB broth to late-log phase and then sequenced as described in Material and Methods section. The results yielded a surprisingly small number of differentially expressed genes (DEGs) between the two strains (Table 5). To evaluate the reliability of the RNAseq results, the expression of these genes was analysed by RT-qPCR. Significant upregulation in the Δ*pssI* mutant was found only for three genes which encoded for PssR (PSA3335_1621), a pyruvate dehydrogenase E1 component beta subunit (PSA3335_1622, *pdhT*) and a pyruvate dehydrogenase E1 component (PSA3335_1624, *pdhQ*) (Table 5). On the other hand, downregulation of any of the genes identified by RNAseq analysis was not observed by RT-qPCR (Table 5). In conclusion, after combination of the results obtained by RNAseq and RT-qPCR, the *pssI* regulon of PSV NCPPB 3335 was restricted to only three genes (*pssR*, *pdhT* and *pdhQ*)

under the conditions tested. The *pdhT* and *pdhQ* genes were also reported to be under the control of the *pssR* homolog in *P. syringae* pv. *syringae* (PSS) strain B728a (28). Interestingly, in PSS, a regulon study also resulted in very small number of genes regulated by AHL QS which are the same loci also determined to be regulated in PSV in this study (28).

Identification of the ET DAPP-PG 735 quorum sensing regulon

It was also of interest to determine the AHL QS regulon in ET therefore transcriptional profiling was also performed via RNAseq comparing the wild type against the ETETOI mutant as described in the Materials and Methods section. DEGs of significance ($p \leq 0.05$) were selected and listed in Table S2. In total, 308 DEGs were identified in the AHL synthase mutant ETETOI mutant, among which 162 loci were down-regulated and 146 up-regulated.

Interestingly, 19% of DEGs (59 genes) were classified as carbohydrate metabolism (Table 6) and, among them, 18 loci of inositol catabolism, which were negatively regulated by EtoI/R. On the other hand, DEGs involved in D-galactarate, D-glucarate and D-glycerate catabolism as well as maltose and maltodextrin utilization were positively regulated by the EtoI/R system. Besides carbohydrate metabolism, EtoI/R regulated genes mostly involved in the metabolism of amino acids, loci involved in membrane transport and in respiration. Furthermore, it was established that menaquinone and phylloquinone biosynthesis, glycerolipid and glycerophospholipid metabolism were influenced by EtoI/R. In addition, 9 transcriptional regulators belonging to the DeoR, IclR, LacI and TetR families were regulated by EtoI/R QS system.

223 In order to corroborate RNAseq results, nine QS-regulated genes were randomly selected
224 and RT-qPCR was carried out with gene-specific primers (Table 3). RNA samples
225 extracted from three biological replicate sets were used as templates for RT-qPCR.
226 Expression patterns determined from RT-qPCR were in good accordance with the
227 expression levels obtained by RNAseq (Figure 3).

228 **Role of PssI/R of PSV NCPPB 3335 *in planta***

229 In order to determine the role of the AHL QS system of PSV NCPPB 335 in virulence,
230 the *ΔpssI* and *ΔpssR* mutants and their respective complemented strains were inoculated
231 in micropropagated and in woody olive plants. In our conditions, no significant
232 differences in knot development among the strains tested were found either in non-woody
233 (micropropagated) or woody olive plants (Figure 3). Additionally, all bacteria reached a
234 similar final population within the knots. It was concluded that PSV NCPPB 3335 AHL
235 QS did not play a significant role in virulence under the conditions tested.

236 ***In planta* role of QS regulated loci of *ET***

237 In order to study the possible role of some ET AHL QS regulated loci in the cooperative
238 interaction with PSV, knock-out mutants in *iolD*, *iotS*, *garL*, *malK*, *gldA* and *hslV* genes
239 were generated by insertion mutagenesis and co-inoculated with PSV in olive plants.
240 Four of these DEGs (*iolD*, *iotS*, *garL* and *malK*) are involved in carbohydrate metabolism,
241 which is the most representative category regulated by AHL QS in ET (see above). The
242 *gldA* and *hslV*, on the other hand, encode for a glycerol dehydrogenase and ATP-
243 dependent protease.

244 As previously established, co-inoculation of PSV with ET significantly increased the size
245 of the olive knot (15, 16). When ET mutants, ETIOTS, ETMALK, ETGLDA and

ETHSLV were co-inoculated with PSV, olive knot size did not show any significant size alteration when compared when co-inoculated with ET wildtype (Figure 4A). Co-inoculation of PSV with ETGARL and ETIOLD, on the other hand, had a significant effect on the olive knot size with approximately a 50% reduction for ETGARL and approximately 20% increase for ETIOLD (Figure 4A). When co-inoculated with ETGARL, the colony forming units (CFU) of PSV in the knot were significantly reduced and resulted in 20% the amount of cells when co-inoculation was performed with the wildtype ET (Figure 4B). A significant reduction in the CFUs of PSV was also observed when co-inoculated with ETIOTS, ETGLDA and ETHSLV regardless that olive-knot size was not significantly affected. In order to further determine the putative role of GarL in PSV-ET interaction, we co-inoculated GFP-labeled PSV with ET wild type or the *garL* mutant constitutively expressing RFP. At 30 dpi knots were visualized in a stereoscopic microscope using GFP and RFP filters (Figure 5A, 5B) and pictures were taken and processed as described in Materials and Methods. Results show that the percentage of PSV population co-localization with ET wild type is under 5%, whereas over 75% of ET co-localize with PSV (Figure 5C). On the other hand, mutation in the ET *garL* gene resulted in a drastic reduction of ET association with PSV, with only 6.6% of the total ET population overlapping PSV. This result, together with the reduced knot size in PSV-ETGARL co-inoculation, indicated that GarL plays a major role in PSV-ET interaction.

Applied and Environmental Microbiology

DISCUSSION

There is a growing need to study interspecies bacterial interactions since it is now becoming evident that most bacteria in the wild live as part of complex communities. Moreover in relation to diseases, reports are beginning to demonstrate that pathogens undergo interactions and communicate with non-pathogenic commensal/resident host microbial flora (11, 29). We have previously reported that the olive knot disease is a model to study interspecies communication and cooperation between a bacterial pathogen and commensal bacteria in a plant disease (14, 15). This cross-communication occurs via cross-feeding/sharing of AHL QS signals whereas the mechanism(s) of cooperation leading to a more aggressive disease is currently not understood and could be due to metabolite(s) sharing and/or metabolic complementarity. In this study, we determined the QS regulons of PSV and ET in order to begin to shed some light in this cooperative interspecies interaction in a plant disease.

Results presented here reveal that all *P. savastanoi* isolates infecting woody plants sequenced so far, harbor an identical content of AHL QS-related genes which consist of an archetypical AHL QS pair designated as *pssI*/*pssR*, and two *luxR* solos. The PssI/R system was firstly reported in strain DAPP-PG 722 (15) and displays 100% identity with PssI/R of strain NCPPB 3335 (studied here). At transcriptional level there is no QS positive feedback loop regulating the AHL synthase gene in PSV NCPPB 3335 (Figure 2A), which is contrast with what occurs in *P. syringae* pv *syringae* (PSS) B728a [50], a strain closely related with PSV from a phylogenetic point of view. It cannot be excluded that one of the two LuxR solos present in PSV genomes might be involved in *pssI* regulation. Moreover, AefR (AHL epiphytic fitness Regulator) positively regulates the

288 *pssI* homolog *ahlI*, in *P. syringae* pv *phaseolicola* NPS3121 (30) and PSS B728a (31); a
289 homolog of AefR is present in PSV genomes and could therefore have a similar function
290 in regulating *pssI* in PSV.
291 *In planta* infection studies revealed that in PSV NCPPB 3335 neither *pssI* nor *pssR* are
292 involved in virulence in the olive plant. It cannot be excluded however that AHL QS in
293 PSV might plays a role in the epiphytic fitness/lifestyle *in planta*; QS has been shown to
294 play a role in epiphytic fitness in PSS as well as other plant-associated bacteria (28, 31-
295 33).
296 We found that wild type PSV NCPPB 3335 produces exclusively C6-AHL, whereas PSV
297 DAPP-PG 722 synthesizes 3-oxo-C6- and 3-oxo-C8-AHLs (15) regardless that the *luxI*
298 homologs are 100% identical; some other factor(s) might be responsible for the
299 generation of different signal molecules. Overexpression of *pssI* in PSV NCPPB 3335
300 yielded 3-oxo-C6- and 3-oxo-C8-HSLs in addition to C6-AHL (Table 2), suggesting that
301 different expression levels between these two strains might explain differences in AHL
302 production. AHLs are synthesized by LuxI using *S*-adenosylmethionine and an acyl
303 group which is provided by an acyl-carrier protein (ACP) (34). We have identified an
304 ACP-encoding gene in the genome of PSV DAPP-PG 722 (locus tag GS14_RS0122650)
305 which is not present in the PSV NCBBP 3335 genome; this locus might be involved in
306 AHL synthesis and consequently lead to a dissimilar AHL profile synthesis between
307 these two PSV strains. We previously reported 3-oxo-C6- and 3-oxo-C8-AHL production
308 by ET DAPP-PG 735 (15) and here we demonstrated the production of four additional
309 types of AHL (C6-, C8-, 3-oxo-C10- and 3-OH-C6-AHLs) using a more sensitive
310 technique. The ability to produce more AHL types by ET increases its ability to cross-

311 talk with bacterial neighbours. The PSV NCPPB 3335 can synthesize three out of the six
312 types of AHL produced by ET indicating possible eavesdropping between PSV and ET
313 via these AHLs. This is in line with our previous study which demonstrated rescue of the
314 PSV QS response of a *pssI* mutant by co-inoculation with ET wild type (15).
315 This study reports the genetic loci regulated by AHL QS in a woody host pathogen of the
316 *P. syringae* complex. Previous reports involve the two *P. syringae* herbaceous pathogens
317 *P. syringae* pv *syringae* (PSS) and *P. syringae* pv. *tabaci* (PST). PSV NCPPB 3335
318 AHL QS regulon consists of only three genetically close loci, namely *pdhT*, *pdhQ* and
319 *pssR*. In PSS strain B728a AHL QS regulates the transcription of only a 9 gene cluster
320 located adjacent to the *ahlR-ahlI* locus which also contains the *pdhT* and *pdhQ* loci (28),
321 whereas in PST strain 11528 over 300 genes were found to be regulated by QS,
322 including *phdT*, *pdhQ* and the *pssR* homologs (35). Despite such a difference in AHL QS
323 regulons among these strains, the transcription of *pdhT*, *pdhQ* and *pssR (ahlR)* is
324 common in all *P. syringae* species and their role in *P. syringae* deserves further attention.
325 (36).
326 QS in *Erwinia* species plays important roles in virulence determinants and secondary
327 metabolite production (37). *E. toletana* is a harmless epiphyte and endophyte and was
328 first isolated from olive knots caused by PSV, and is now a model to study multispecies
329 interactions with PSV (14). ET DAPP-PG 735 possesses two canonical AHL QS systems,
330 designated as EtoI/R and TolI/R. Prior to the availability of the genome sequence, AHL
331 QS signals produced by ET were initially only attributed to EtoI (15). Here we report that
332 promoter activities of *tolI/R* in ET, ETTOLI and ETETOI were very low and were barely
333 detectable *in planta* and were found to be repressed by the plant apoplast mimic medium,

suggesting that *tolI/R* is stringently regulated and might need a yet unidentified stimulus to be expressed. It is common that two or more AHL QS systems coexist in one bacterium and many of these are interconnected in their regulation (38-43). The uniqueness in *E. toletana* is that one system is stringently regulated probably requiring, in addition to cell-density, an environmental stimulus in order to be activated and/or de-repressed.

In ET, 308 genes were found to be regulated by EtoI/R controlling diverse functions such as membrane transport, protein metabolism, respiration, stress response, cell division and cell cycle. Interestingly, 59 loci were involved in metabolism of carbohydrates including inositol, D-galactarate, D-glucarate, maltose and maltodextrin indicating that it plays an important role in carbon resource acquisition. It was therefore of interest to study whether any of these carbohydrate metabolic pathways play a role in interspecies interactions and cooperation with PSV. As shown in Figure 4, when co-inoculated with several ET mutants in these pathways, PSV reached lower population densities, indicating that *iotS*, *garL*, *gldA* and *gslV* ET genes play a role in PSV-ET cross-communication. IN addition, co-inoculation of the ET *garL* mutant with PSV resulted in a significantly smaller olive knot. The alpha-dehydro-beta-deoxy-D-glucarate aldolase GarL catalyzes the cleavage of both 5-keto-4-deoxy-D-glucarate and 2-keto-3-deoxy-D-glucarate to pyruvate and tartronic semialdehyde (44). GarL is involved in D-galactarate, D-glucarate and D-glycerate catabolism synthesizing D-glycerate from galactarate. This demonstrates that ET-PSV cross-communication also occurs through some reactions of primary metabolism that not only affect the growth of PSV *in planta*, but also its virulence.

Applied and Environmental Microbiology

356 In summary, this work further demonstrated the role of AHL QS in the olive knot as well
357 as metabolic interaction. This therefore further highlights the olive knot as a good model
358 to study bacterial interspecies interactions in planta of a plant disease.
359

MATERIALS AND METHODS

Bacterial strains, media, growth conditions and recombinant DNA techniques

Bacterial strains used in this study are listed in Table 1. PSV and ET were grown at 28 °C and *Escherichia coli* was grown at 37 °C in Luria-Bertani (LB) medium (45) and Super Optimal Broth (SOB) (46). Solid and liquid media were amended when required with the appropriate antibiotic. Antibiotic concentration used were: kanamycin (Km) 10 µg ml^{-1} for PSV and 50 µg ml^{-1} for *E. coli*, gentamycin (Gm) 10 µg ml^{-1}, ampicillin (Ap) 400 µg ml^{-1} for PSV and 100 µg ml^{-1} for *E. coli*; and tetracycline 10 µg ml^{-1}.

All recombinant DNA techniques including restriction digestion, and agarose gel electrophoresis, purification of DNA fragments and ligations with T4 DNA ligase were performed as previously described (47). Plasmids were purified by using EuroGold columns (EuroClone, Italy) and were sequenced by Macrogen Europe (Amsterdam, NL) when necessary.

Construction of bacterial strains

Plasmids and oligonucleotides used in this study are listed in Tables 2 and 3, respectively. PSV NCPPB 3335 *pssI* (PSA3335_1620) and *pssR* (PSA3335_1621) mutants were generated by allelic interchange. DNA fragments of approximately 1 kb corresponding to the upstream and downstream flanking regions of the gene to be deleted were amplified in three rounds of polymerase chain reaction (PCR) using Expand High Fidelity polymerase (Roche Applied Science, Mannheim, Germany). Restriction sites for *Hind*III were included in the primers as previously described (48). The resulting products, consisting on upstream and downstream flanking regions separated by the *Hind*III restriction site, were cloned into pGEMT-Easy (Promega, Madison, WI, U.S.A.) and

383 sequenced to discard mutations. Next, the kanamycin resistance gene *nptII* was extracted
384 by enzyme restriction from pGEMT-KmFRT- *Hind*III (49) and cloned in the plasmids
385 mentioned above to generate pECP10-Km and pECP11-Km (Table 2). All the plasmids
386 generated for the construction of PSV NCPPB 3335 mutants were suicide vectors in PSV.
387 Plasmids were transferred to NCPPB 3335 by electroporation (17) and transformants
388 were selected in LB-Km plates. To select the allelic interchange (double recombination
389 event) and discard plasmid integration (single recombination event), individual colonies
390 were replicated into LB-Ap plates and Ap^R colonies were discarded. Southern blot
391 analyses were carried out to confirm single integration in the correct position in PSV
392 genome.
393 Mutation of selected genes in ET was performed via a single homologous recombination
394 event with the use of pKNOCK-Km suicide delivery system as previously described (50)
395 generating mutants of ETIOLD, ETIOTS, ETGARL, ETMALK, ETGLDA, ETHSLV,
396 ETTOLI and ETTOLR. Briefly, internal fragments from *iolD* (G200_RS0103425), *iotS*
397 (G200_RS0119945), *garL* (G200_RS0124305), *malK* (G200_RS0114460), *gldA*
398 (G200_RS0114990), *hslV* (G200_RS0113655), *tolI* (G200_RS0118785) and *tolR*
399 (G200_RS0118780) of ET were amplified using the primers listed in Table 3 and cloned
400 in conjugative suicide vector pKNOCK-Km. The generated plasmids having internal
401 fragments from selected genes were transformed into *E. coli* S17-1 λpir and delivered to
402 ET for its homologous recombination. Km^R colonies were verified by PCR analysis
403 followed by sequencing of the targeted gene to confirm the generation of ET mutants.
404 **AHL extraction and characterization**

405 Bacterial strains were grown overnight in LB broth (final volume 100 ml); cells were
406 then removed by centrifugation and the supernatant was used to purify AHLs. Spent
407 supernatants were filtered (pore diameter 0.45 µm), mixed with one volume of 0.1%
408 acetic acid (v/v) in ethyl acetate and incubated under shaking conditions for 30 minutes.
409 The organic phases were dried at room temperature. The AHLs produced by each strain
410 were identified from the organic extracts of spent supernatants by liquid chromatography-
411 electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) as we described
412 previously (51). As an example of this analysis, the ion chromatograms of an AHL
413 standard and the *E. toletana* wild type sample is provided in Figure S3.

414 **Construction of plasmids and reporter assays**

415 For the complementation of PSV Δ*pssI* and Δ*pssR* mutant strains, the entire open reading
416 frames of each gene and their corresponding promoter and transcriptional terminator
417 regions were amplified by PCR using Expand High Fidelity polymerase (Roche Applied
418 Science, Mannheim, Germany) and cloned into pGEMT-Easy (Promega, Madison, WI,
419 USA). After sequencing to discard mutations, the fragments were directionally subcloned
420 into pBBR:MCS5 yielding pBBR:*pssI* and pBBR:*pssR*.

421 DNA fragments of 338 and 352 bp containing *pssI* and *pssR* promoter regions,
422 respectively, were amplified by PCR using oligonucleotides listed in Table 3 and cloned
423 into pMP220 (52). The resulting plasmid *lacZ* transcriptional fusions were transferred to
424 PSV by electroporation and β-galactosidase activity was measured as described
425 previously (45). Bacteria were grown in LB broth amended with 10 µg ml^{-1} tetracycline
426 at an initial OD_{600nm} of 0.3 and β-galactosidase activity was measured throughout the
427 growth curve.

Promoter regions of *etoI*, *etoR*, *tolI* and *tolR* ET genes were amplified by PCR using the oligonucleotides listed in Table 3 and cloned in the vector pBBR:GFP (53) in order to be transcriptionally fused to a promoterless *gfp* gene. The resulting plasmids were transformed by electroporation into ET strains (54) and gene promoter activity was determined as the amount of GFP fluorescence measured in the late log phase at 510nm on a microplate reader (Perkin Elmer EnVision 2104). The expression of *tolI* and *tolR* was also analyzed by RT-qPCR in King's B and Hrp-inducing medium as reported previously (48). The *etoI* and *tolI* promoter activities were measured *in vivo* in mixed PSV-ET infections. Ten plants were inoculated with each of the three combinations: PSV and ET expressing a promoterless GFP (negative control), PSV and ET-pBBR:P_{etoI}-GFP, and PSV and ET-pBBR:P_{tolI}-GFP. The presence/absence of fluorescence was verified using a stereoscopic microscope (Leica MZ FLIII; Leica Microsystems, Wetzlar, Germany).

RNA extraction, RNAseq and analysis

Ribopure bacteria RNA isolation kit (Ambion Inc., Austin, TX, U.S.A.) was used for total RNA extraction from three biological replications. Bacteria were grown in LB until the onset of stationary phase and about 2 x 10^9 cells were collected for RNA extraction following the manufacturer's instructions. Library preparation and transcriptome sequencing were performed by IGA Technology Services Srl (Udine, Italy). Briefly, libraries were constructed with TruSeq Stranded mRNA Sample Prep kit (Illumina, San Diego, CA) and single-end sequencing was carried out on HiSeq2500 (Illumina, San Diego, CA). Illumina adapters, lower quality bases and poly-A tails were removed using ERNE (55). Software and tools for *de novo* assembly and comparisons were performed as

previously described (56-58). The false discovery rate (FDR) with a significance level of ≤0.05 and with a minimum fold change set as the threshold were used to judge the significance of gene expression difference. Reads obtained from adapter removal were aligned against GCA_000336255.1 and GCA_000164015.2 reference genome assemblies. Features counts produced by RNA-seq were normalized and analyzed with DeSeq2 software (http://dx.doi.org/10.1186/s13059-014-0550-8) to calculate differential expression values (log_2 of the fold change LFC) and raw p-values. To select differentially expressed genes, genes raw p-values were corrected for multiple testing using with the false discovery rate (FDR) method (59). Final selection was based on genes with FDR ≤0.05. The original RNAseq data has been submitted in the Sequence Read Archive (SRA) as submission number SUB3743389

Validation of RNAseq data using qRT-PCR

Quantitative real-time PCR was performed on CFX96 Touch qPCR system (Bio-Rad, Hercules, CA, USA) to validate expression patterns from transcriptome analysis. cDNA was generated following the manufacturer's protocol of Reverse Transcription system kit (Promega, Madison, WI, USA) starting with 1-2 µg of purified RNA as input. Diluted with RNase-free water, the synthesized cDNA samples were adjusted to 25 $ng \cdot \mu L^{-1}$ and were measured by Nano Drop 2000 (Thermo scientific, Wilmington, DA, USA). In each reaction, 2 µL of cDNA template was mixed with GoTaq qPCR Master Mix kit (Promega, Madison, WI, USA) and specific primers (Table 3) to a final volume of 12 µl. qPCR primer designing was performed with free online software following the instructions of Brenda Thornton and Chandak Basu (60). Each reaction was carried out initially with 2 min at 95 °C, followed by 45 cycles of PCR (95 °C, 15 s; 60 °C, 30 s). The relative

transcript abundance was calculated using the cycle threshold (ΔΔCt) method (61). Transcriptional data were normalized to the *gyrA* (for PSV) or *recA* (for ET) housekeeping genes.

In planta experiments

Olive plants were micropropagated and inoculated as detailed previously (62). Briefly, micropropagated olive plants were wounded by excision of an intermediate leaf and infected in the stem wound with a bacterial suspension under sterile conditions. For this purpose, bacterial lawns were grown for 48h on LB plates, washed twice with 10 mM $MgCl_2$ and resuspended in 10 mM $MgCl_2$ to an approximate concentration of 10^8 $CFU \cdot mL^{-1}$. Suspension of PSV alone or mixed with ETIOLD, ETIOTS, ETGARL, ETMALK, ETGLDA and ETHSLV respectively in 1:1 (vol:vol) ratio were prepared. Plants were inoculated with approximately $5x10^3$ total CFU and kept in a growth chamber for 30 days, as previously described (62). The morphology of the knots was observed with a stereoscopic microscope 30 days post-inoculation (dpi) (Leica MZ FLIII; Leica Microsystems, Wetzlar, Germany), also equipped with a 100 W mercury lamp, a GFP2 filter (excitation 480/40 nm; emission 510LP nm) and a red fluorescent protein (RFP) filter (excitation 546/10 nm; emission 570LP nm). For the quantification of green (GFP-tagged PSV) and red (RFP-tagged ET and ETGARL strains) pixels, two pictures per knot (corresponding to the front and back sides of the tumour) were taken with each the GFP2 and RFP filters. Pictures were transformed to 8-bits images and overlapped with Fiji ImageJ (https://imagej.net/Fiji) using the Image correlator plugin. The number of green pixels overlapping red pixels, indicating the population of PSV that co-localize with ET/ETGARL, was determined for both the front and the back sides of each knot and an

497 average per knot was calculated. An identical procedure was used to determine the
498 percentage of ET or ETGARL population that co-localize with PSV. Bacteria were
499 recovered from the knots using a mortar and pestle containing sterile $MgCl_2$ 10 mM.
500 Serial dilutions were plated on LB plates supplemented with the corresponding antibiotic
501 when required. Knots were 3D scanned and the knot size determined using the Neftabb
502 Basic 5.2 software.
503 The virulence of PSV and its derived mutants and complemented strains was also
504 analysed on 1-year old olive plants on 1-year old olive plants (*Olea europaea*) derived
505 from a seed originally collected from a cv. Arbequina plant as detailed before (17, 63, 64).
506 Morphological changes scored at 90 dpi were captured with a high-resolution camera
507 Canon D6200 (Canon Corporation, Tokyo, Japan). The knot volume was calculated from
508 a minimum of three representative knots as described previously (15, 65).
509

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513
514

Figure Legends

Figure 1. Gene arrangement of quorum sensing system elements in the genomes of PSV NCPPB 3335 and ET DAPP-PG 735. (A) *pssI* and *pssR* represent a canonical *luxI*/*luxR* gene pair, whereas *luxR2* and *luxR3* correspond to orphan *luxR* homologs in PSV NCPPB 3335. (B) *etoI*/*etoR* and *tolI*/*tolR* represent two canonical *luxI*/*luxR* gene pairs of ET DAPP-PG 735. Codes above arrows correspond to locus tags

Figure 2. Promoter activities of PSV and ET quorum sensing genes. (A) β-galactosidase activity of *pssI* promoter fusion to *lacZ* measured in PSV NCPPB 3335, Δ*pssI* and Δ*pssR* at log (4 hours incubation) and stationary phase (10 hours incubation). PSV harboring a promoterless *lacZ* (empty pMP220 plasmid) was included as a control. Asterisks indicate a significant difference (student's *t* test, $P < 0.05$) in promoter activity in stationary phase compared to log phase (B) GFP fluorescence of *etoI*, *etoR tolI* and *tolR* fusions to *gfp* measured in ET, ETTOLI and ETETOI backgrounds. GFP fluorescence was normalized to OD_{600}. Bars represent the average of three independent replications ± the standard deviation

Figure 3. Evaluation of RNAseq-based expression patterns of ET using RT-qPCR. The expression patterns of randomly selected genes were analyzed by RT-qPCR to validate RNAseq results. The values of fold difference were average of three biological replicates which were calculated by using comparative quantification method. Log_2 ratio of obtained values was compared with log_2 ratio of (ETETOI/ET) FPKM values.

Figure 4. Role of ET AHL QS loci in the PSV-ET cooperation *in planta*. (A) Size of the knots induced in micropropagated olive plants at 30 dpi by PSV in combination with ET

strains. (B) CFU of PSV and (C) CFU of ET recovered from knots. Bars indicate the average of, at least, three knots ± standard deviation.

Figure 5. Knots developed at 30 dpi in micropropagated olive plants after co-inoculation of GFP-labelled PSV with RFP-labelled ET or ETGARL. (A) Co-inoculation using GFP-labelled PSV and RFP-labelled ET. (B) Co-inoculation using GFP-labelled PSV and RFP-labelled ETGARL. (C) Percentage of the PSV and ET/ETGARL populations co-localization within the knot. Bars represent the average of six independent knots ± standard deviation.

553 **Table 1.** Bacterial strains used in this study
554

Bacterial Strains	Relevant characteristics	Source
Escherichia coli		
DH5α	F– Φ80*lacZ*ΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1	Invitrogen-LifeTechnologies
S17-1λpir	Km^R, recA, pro, hsdR, RP4-Tc::Mu-Km::Tn7, pir	(66)
Erwinia toletana		
DAPP-PG 735	Wild type	(15)
ETETOI	Deletion *etoI* mutant of ET DAPP-PG735	(15)
ETETOR	Deletion *etoR* mutant of ET DAPP-PG735	(15)
ETTOLI	Deletion *tolI* mutant of ET DAPP-PG735	This study
ETTOLR	Deletion *tolR* mutant of ET DAPP-PG735	This study
ETIOLD	Deletion *iolD* mutant of ET DAPP-PG735	This study
ETIOTS	Deletion *iotS* mutant of ET DAPP-PG735	This study
ETGARL	Deletion *garL* mutant of ET DAPP-PG735	This study
ETMALK	Deletion *malK* mutant of ET DAPP-PG735	This study
ETGLDA	Deletion *gldA* mutant of ET DAPP-PG735	This study
ETHSLV	Deletion *hslV* mutant of ET DAPP-PG735	This study
ETETOI-pBBR:*etoI*	ETETOI complemented with pBBR:*etoI*	This study
ETTOLI-pBBR:*tolI*	ETTOLI complemented with pBBR:*tolI*	This study
Pseudomonas savastanoi pv. *savastanoi*		
NCPPB 3335	Wild type	(17)
Δ*pssI*	Deletion *pssI* mutant of NCPPB 3335 (Km^R)	This study
Δ*pssR*	Deletion *pssR* mutant of NCPPB 3335 (Km^R)	This study
Δ*pssI*-pBBR:*pssI*	Δ*pssI* complemented with pBBR:*pssI*	This study
Δ*pssR*-pBBR:*pssR*	Δ*pssR* complemented with pBBR:*pssR*	This study

555

556 **Table 2** Plasmids used in this study

557

pGEM-T Easy	Cloning vector; Amp^R	Promega
pKNOCK-Km	Conjugative suicide vector; Km^R	(50)
pKNOCK- IOLD	Internal PCR iolD fragment of ET DAPP-PG 735 cloned in pKNOCK-Km; Km^R	This study
pKNOCK- IOTS	Internal PCR iotS fragment of ET DAPP-PG 735 cloned in pKNOCK-Km; Km^R	This study
pKNOCK- GARL	Internal PCR garL fragment of ET DAPP-PG 735 cloned in pKNOCK-Km; Km^R	This study
pKNOCK- MALK	Internal PCR malK fragment of ET DAPP-PG 735 cloned in pKNOCK-Km; Km^R	This study
pKNOCK- GLDA	Internal PCR gldA fragment of ET DAPP-PG 735 cloned in pKNOCK-Km; Km^R	This study
pKNOCK- HSLV	Internal PCR hslV fragment of ET DAPP-PG 735 cloned in pKNOCK-Km; Km^R	This study
pECP10-Km	pGEM-T Easy derivative containing 1kb on each side of the *pssI* (PSA3335_1621) gene from NCPPB 3335 interrupted by the kanamycin resistance gene *nptII* (Ap^R, Km^R)	This study
pECP11-Km	pGEM-T Easy derivative containing 1kb on each side of the *pssR* (PSA3335_1622) gene from NCPPB 3335 interrupted by the kanamycin resistance gene *nptII* (Ap^R, Km^R)	This work
pGEMT-KmFRT-HindIII	Contains KmR from pKD4 and HindIII sites (ApR KmR)	This work
pBBR:*pssI*	pBBR1MCS-5-derivative containing the PSV NCPPB 3335 *pssI* and its promoter region (352 bp) flanked by *Eco*RI and *Xba*I restriction sites (Gm^R)	This work
pBBR:*pssR*	pBBR1MCS-5-derivative containing the PSV NCPPB 3335 *pssR* and its promoter region (435 bp) flanked by *Eco*RI and *Xba*I restriction sites (Gm^R)	This work
pMP220	Promoter probe vector, IncP, LacZ; Tc^R	(52)
pMP220-P*pssI*	Transcriptional fusion of PSV *pssI* promoter to *lacZ*	This work
pLRM1-GFP	Overexpression of GFP from pBBRMCS5	(67)
pBBR:RFP	pBBRMSC5 containing RFP	(53)
pBBR:GFP	pBBRMSC5 containing a promoterless GFP	(53)
pBBR:P*etoI*-GFP	Transcriptional fusion of ET *etoI* promoter to GFP	This work
pBBR:P*etoR*-GFP	Transcriptional fusion of ET *etoR* promoter to GFP	This work
pBBR:P*tolI*-GFP	Transcriptional fusion of ET *tolI* promoter to GFP	This work
pBBR:P*tolR*-GFP	Transcriptional fusion of ET *tolR* promoter to GFP	This work
pBBR:*etoI*	pBBR1MSC-5 containing *etoI*, Described as pBBRTolI in previous publication	(15)
pBBR:*tolI*	pBBR1MSC-5 containing *tolI*	This work

558

559

560 **Table 3** Primers used for cloning purposes
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Primers used for cloning purposes		
Plasmid	Primer name	Primer sequence
pKNOCK- IOLD	iolD_pnkFw	AGATCTCACCAGATTCCGTTTGCCG
	iolD_pnkRev	CTCGAGCTGTTGTAATCCCTGGTGCG
pKNOCK- IOTS	iotS_pnkFw	AGATCTGCTGACCGATAAAATGGCGT
	iotS_pnkRev	CTCGAGACCAATCGCCATTTCATCGT
pKNOCK- GARL	garL_pnkFw	AGATCTGTCCACCTTGCAACGAACC
	garL_pnkRev	CTCGAGGAGCTGGGTTTCGATTTGCA
pKNOCK- MALK	malK_pnkFw	AGATCTATTGGTCGCACGCTGGTC
	malK_pnkRev	CTCGAGCGATGGCCTTGTTAGTGACC
pKNOCK- GLDA	gldA_pnkFw	AGATCTCCGATGAAGGGGTGTTTGAA
	gldA_pnkRev	CTCGAGCCAGACCGCCATTCTCAAAG
pKNOCK- HSLV	hslV_pnkFw	AGATCTGGTCATCTGGTTAAAGCCGC
	hslV_pnkRev	CTCGAGCACCTGAACCGATGGCAATA
pKNOCK-tolI	muttolIFw	GGATCACTGTGCCCTTTA
	muttolIRev	TTATCCTCAGAGTGAATCAGCC
pKNOCK-tolR	muttolRFw	TACGCGACCTGAGACGCATC
	muttolRRev	ATTTTACGATTTCCAGCTCGCG
pBBR:P*tolI*-GFP	PtolIFw	CAGAGATCTCGCTGATTC
	PtolIRev	CGAATTCCGCCAACAACGA
pBBR:P*tolR*-GFP	PtolRFw	AATCGTGGATCCGCGG
	PtolRRev	CGAATTCACCACACCAG
pBBR:P*etoI*-GFP	PetoIFw	TTAGATCTAAATCACGTAACAAC
	PetoIRew	ATTCGAATTCATATCAAA
pBBR:P*etoR*-GFP	PetoRFw	CAGATCTGCTCTTCCTGTAATGGGA
	PetoIRew	CGAATTCACATTTGCCTGACCTCAA
pBBR:*pssI*	pssI_F-331	TCTAGATCGCTCTGATCCTGATGAGTG
	pssI_R924	GAATTCCTCATCCGCTTCCATGACC
pBBR:*pssR*	pssR-F-417	TCTAGAAGACGCTCGACGATGTCG
	pssR_R993	GAATTCTTGCAATCGATCATCACGG
pBBR:*tolI*	tolIFw	GTCTCGAGCAAATCTGCTGATGCCGC
	tolIRev	GGACTAGTGCCTGGCTGCTGATTACTTT
pMP220-P*pssI*	pssI_F-279	ACTCATGGAGATCTGGCAGAGATTTCGTGTTGGG
	pssI_R35	ACTCATGGGGTACCGTAACGGGCATCGTCGTG
pBBR:*pssI*	pssI_F-331	TCTAGATCGCTCTGATCCTGATGAGTG
	pssI_R924	GAATTCCTCATCCGCTTCCATGACC
pBBR:*pssR*	pssR-F-417	TCTAGAAGACGCTCGACGATGTCG
	pssR_R993	GAATTCTTGCAATCGATCATCACGG
Primers used for the construction of *pssI* and *pssR* mutants		
pssR	PssR_F-1008	CATTCCAGTGCTCCTTGAGC
	TAPssR_R3	AAGCTTGACTCACTATAGGGGCTTTCACGGTACGAACCTC
	TDPssR_R739	CCCTATAGTGAGTCAAGCTTCCATCAACATGGGCATGG
	PssI_F-332	CCTGATGAGTGTGTGCATCG
pssI	TAPssI_R4	CCCTATAGTGAGTCAAGCTTCATGCATAGCGCTGCCTG
	PssI_F-983	GATATCGGCGTTGATGTCCTG
	TDPssI_F680	CCCTATAGTGAGTCAAGCTTCATGCATAGCGCTGCCTG
	PssR_F-280	TGCGCTGTTCATCACTACTCC
Primers used in the qPCR experiments		
Gene ID	Gene function	Primer sequence
E. toletana genes		
G200_RS0108970	PTS lactose transporter subunit IIB	F: ACTCTGCGTATGTGGCTG R: TCGCTGGCATCTGAGGTT
G200_RS0124540	Recombinase RecA	F: CAGGCGATGCGTAAACTGG

		R: GGCGAACAGAGGCGTAGA
G200_RS0112020	Sigma-fimbria uncharacterized paralogous subunit	F: CCTCGGTGTTGCCTCTTC R: CCATTGCCTGCTGAACCC
G200_RS0112675	SulP family transporter	F:GTGTATGTGGTGGCGGTG R: CACTGAGGTAATCGGCAAGC
G200_RS0113655	ATP-dependent protease HslV	F: GTAGTGATTGGCGGCGATG R: CCACAGCGGCTTTAACCAG
G200_RS0114400	Conjugal transfer protein TraF	F: GGCTACACCGATACTTACCAGA R: CACGATAACCACCACGCAA
G200_RS0103275	HlyD family secretion protein	F: AAACCCGCATCAACCCAC R: ATCACGCTTCACCTCATCCT
G200_RS0103290	Hemagglutinin	F: CCTGTTGCTGGGTTCATTGTT R: GTGGTGGTAGCCGAGGTTT
G200_RS0123635	Transcriptional regulator, TetR family	F: GCAGTCACAGGATGCGATTC R: TGAGCCATACACCAGCGATAG
G200_RS0123645	TIM-barrel signal transduction protein	F: CGCTGAAACCGCACTGAAA R: GCCGTAGAAACCATCGCAAA
G200_RS0118785	*tolI*	F: TGGAGAAGGCTGGTCTATTC R: GCATTAAAGGGCACAGTGAT
G200_RS0118780	*tolR*	F: TAATGCGTCTGAAACTGGTC R: CGACATATTTCTTCTGCCGA
P. savastanoi pv. **savastanoi** genes		
PSA3335_1622	Pyruvate dehydrogenase E1 component, beta subunit	F: TCAAGGAGCACTGGAATGTCG R: TCTTCAAGGGATGGAAACGATT
PSA3335_1624	Pyruvate dehydrogenase E1 component	F: CGATACCGTGCTGTGTGTCT R: GATCAGGGTGCGGGTAGTTC
PSA3335_1621	LuxR transcriptional regulator	F: ACTGCCCACCGTTGAAGATAA R: CATAAGATTTCAGCCAGGAGTCG
PSA3335_2315	Putative hydrocarbon oxygenase	F: TGCCGTTCTTCCTGGCTTA R: ACCCGTCATTCATCCACCG
PSA3335_4742	Urocanate hydratase	F: AGCGGGCATTCCTACCTTC R: AGAACAACGGGCGGATGTA
PSA3335_1620	Homoserine lactone synthase	F: CACTGACCGAAATGCTGCTGT R: TTGCTGACCACCGTGATGAT
PSA3335_4623	Copper chaperone	F: GACTCAAGCGATCAAGAACGATG R: CTGCTCGGGTGACAGACTG
PSA3335_2048	Hypothetical protein	F: AATACCACCGCATCGACGAA R: TCACGCCGTTGACCAGAAA
PSA3335_0454	Malonate decarboxylase delta subunit	F: TTCGCCAGGCAAGCTATCAA R: TCCTCGAAGCCCTGATCCA
PSA3335_2054	Hypothetical protein	F: TGAGCATCTACAGGCTTCGGA R: CATGTTGATAAGGAATGAGGTTCG
PSA3335_4121	Pectin lyase precursor	F: CCAAGGTGCAGGACTGTTCA R: GATACGGGCGAAGGTGTTGT

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565 **Table 4**. Quantification of AHLs produced by PSV NCPPB 3335 and ET DAPP-PG 735[a]
566

	C6-AHL	C8-AHL	3-oxo-C6-AHL	3-oxo-C8-AHL	3-oxo-C10-AHL	3-OH-C6-AHL
PSV	+	-	-	-	-	-
Δ*pssI*	-	-	-	-	-	-
Δ*pssI*-pBBR:*pssI*	+++	+	+++	++	-	-
ET	+++	+	+++	+++	+	++
ETETOI	-	-	-	-	-	-
ETETOI-pBBR:*etoI*	+++	+++	+++	+++	+++	+++
ETTOLI	+++	+	+++	+++	-	++
ETTOLI-pBBR:*tolI*	+++	+	+++	+++	-	++

567
568 [a] -, no production; +, relative peak area <100,000; ++, relative peak area between 100,000 and 1,000,000;
569 +++, relative peak area >1,000,000
570

Table 5. Genes regulated by *pssI* in PSV NCPPB 3335

Locus tag[a]	Gene[b]	Gene product	RNAseq[c]	RT-qPCR[c]
Upregulated				
PSA3335_1622	*pdhT*	Pyruvate dehydrogenase E1 component, beta subunit	3.27	3.6
PSA3335_1624	*pdhQ*	Pyruvate dehydrogenase E1 component	2.97	2.32
PSA3335_1621	*pssR*	LuxR transcriptional regulator	1.44	3.95
Downregulated				
PSA3335_4623	UN	Copper chaperone	-1.07	-0.82
PSA3335_4121	UN	Pectin lyase precursor	-0.92	0.52

[a]Upregulated or downregulated genes in the Δ*pssI* mutant according to RNAseq data.
[b]UN, unnamed
[c]The $\log_2$ (fold change) obtained in the RNAseq and RT-qPCR experiments are represented. The fold change refers to the ratio of the average expression obtained in the Δ*pssI* mutant versus the wild type strain in three biological replicates. Genes which QS-dependent expression was corroborated by RT-qPCR are underlined

580 **Table 6**. Genes regulated by *etoI* in ET DAPP-PG 735 classified as carbohydrates
581 metabolism
582
583

Gene ID	log_2(ETEOI/ET)	FDR	Gene product
Inositol catabolism			
G200_RS0101695	2.56366058	1.13E-09	Major myo-inositol transporter IolT
G200_RS0103410	2.81622077	3.38E-45	Inosose dehydratase IolE
G200_RS0103415	3.051765216	2.74E-47	Glyceraldehyde-3-phosphate ketol-isomerase IolH
G200_RS0103420	3.612129551	3.41E-57	Myo-inositol 2-dehydrogenase 1 IolG
G200_RS0103425	3.034782963	1.24E-11	Epi-inositol hydrolase IolD
G200_RS0103430	2.455264684	4.22E-08	5-keto-2-deoxygluconokinase IolC
G200_RS0103435	1.82595615	7.10E-18	Transcriptional regulator of the myo-inositol catabolic operon IolR
G200_RS0103440	2.322930823	6.16E-29	5-deoxy-glucuronate isomerase IolB
G200_RS0103445	2.343048715	1.92E-08	Methylmalonate-semialdehyde dehydrogenase IolA
G200_RS0103450	2.393433177	3.58E-08	Inosose isomerase IolI
G200_RS0103485	2.265947451	7.57E-22	Inosose dehydratase
G200_RS0103490	1.606575527	5.42E-13	Myo-inositol 2-dehydrogenase
G200_RS0109945	2.054104613	1.56E-06	Myo-inositol 2-dehydrogenase
G200_RS0111735	2.826752946	1.30E-21	Major myo-inositol transporter IolT
G200_RS0119935	2.36064358	6.17E-31	Inositol transport system permease protein
G200_RS0119940	2.922693363	5.94E-43	Inositol transport system ATP-binding protein
G200_RS0119945	2.723773939	6.09E-34	Inositol transport system sugar-binding protein
G200_RS0120045	2.507147476	1.44E-09	Myo-inositol 2-dehydrogenase 2
D-galactarate, D-glucarate and D-glycerate catabolism			
G200_RS0114355	-1.471923639	1.25E-06	MFS transporter
G200_RS0124280	-2.146858379	7.57E-39	D-galactarate dehydratase GarD
G200_RS0124290	-2.155286292	3.22E-65	D-glucarate permease
G200_RS0124295	-1.762097381	2.90E-22	Glucarate dehydratase GudD
G200_RS0124300	-1.801096855	3.95E-16	Glucarate dehydratase GudD
G200_RS0124305	-1.841655614	7.57E-39	2-dehydro-3-deoxyglucarate aldolase GarL
G200_RS0124320	-1.921850417	2.06E-49	Glycerate kinase
G200_RS25820	-2.073060541	9.97E-53	3-hydroxyisobutyrate dehydrogenase GarR
Maltose and Maltodextrin catabolism			
G200_RS0105520	-1.474187006	1.51E-22	PTS system, maltose and glucose-specific IIABC component
G200_RS0114455	-2.146215061	6.10E-08	Maltose/maltodextrin high-affinity receptor LamB
G200_RS0114460	-3.460976388	1.17E-46	Maltose/maltodextrin transport ATP-binding protein MalK

Applied and Environmental Microbiology

G200_RS0114465	-3.432632153	3.66E-74	Maltose/maltodextrin ABC transporter, substrate binding periplasmic protein MalE
G200_RS0114470	-1.356395147	1.92E-06	Maltose ABC transporter permease MalF
Other carbohydrates metabolism			
G200_RS0102345	-1.326068817	1.39E-19	6-phospho-beta-glucosidase
G200_RS0120860	-1.385897754	2.78E-07	PTS beta-glucoside transporter subunit EIIBCA
G200_RS0109880	1.195365256	3.02E-14	Beta-glucuronidase
G200_RS0108005	1.553737025	2.47E-29	Alcohol dehydrogenase
G200_RS0118365	1.430810069	6.79E-14	Pyruvate formate-lyase
G200_RS0108900	1.148093171	1.24E-10	Deoxyribose-phosphate aldolase
G200_RS0116155	1.055489576	4.73E-12	Ribokinase
G200_RS0109855	1.1278524	1.96E-13	Mannonate dehydratase
G200_RS0102390	2.327906926	3.00E-11	Gluconate 2-dehydrogenase, membrane-bound, flavoprotein
G200_RS0102395	2.004607764	1.22E-17	Gluconate 2-dehydrogenase, membrane-bound, gamma subunit
G200_RS0119505	1.507781586	2.74E-15	Ribose ABC transport system, periplasmic ribose-binding protein RbsB
G200_RS0118360	1.344548034	3.43E-28	Pyruvate formate lyase 1-activating protein PflA
G200_RS0121040	1.68097694	2.43E-04	Aerobic glycerol-3-phosphate dehydrogenase GlpD
G200_RS0121025	-1.369452767	7.02E-22	Glucose-1-phosphate adenylyltransferase GlgC
G200_RS0121030	-1.413808857	4.42E-26	Glycogen synthase GlgA
G200_RS0108965	1.651181716	2.59E-16	6-phosphofructokinase
G200_RS0105430	-1.010271981	3.98E-16	Aconitate hydratase AcnA
G200_RS0114595	-1.302381077	1.85E-17	Malate synthase
G200_RS0101545	-2.502124169	2.13E-42	L-lactate dehydrogenase
G200_RS0109845	1.012221802	6.78E-09	MFS transporter LacY
G200_RS0118000	1.499549769	9.33E-05	6-phosphogluconolactonase
G200_RS0100900	-1.050323621	2.49E-14	DUF485 domain-containing protein
G200_RS0100905	-1.143638292	2.68E-11	Cation/acetate symporter ActP
G200_RS0121020	-1.529478972	2.20E-39	Glycogen debranching enzyme
G200_RS0113990	1.292475653	5.30E-11	PTS sugar transporter subunit IIB
G200_RS0113995	1.209666009	1.43E-15	Putative carbohydrate PTS system, IIA component
G200_RS0114000	1.458640254	1.69E-13	Putative transcriptional regulator of unknown carbohydrate utilization cluster, GntR family
G200_RS0104280	-1.044495518	3.39E-06	Alpha/beta hydrolase

Gene ID	**log_2(ETEOI/ET)**	**FDR**	**Gene product**
Inositol catabolism			
G200_RS0101695	2.56366058	1.13E-09	Major myo-inositol transporter IolT
G200_RS0103410	2.81622077	3.38E-45	Inosose dehydratase IolE

G200_RS01034155	3.051765216	2.74E-47	Glyceraldehyde-3-phosphate ketol-isomerase IolH
G200_RS01034200	3.612129551	3.41E-57	Myo-inositol 2-dehydrogenase 1 IolG
G200_RS01034255	3.034782963	1.24E-11	Epi-inositol hydrolase IolD
G200_RS01034300	2.455264684	4.22E-08	5-keto-2-deoxygluconokinase IolC
G200_RS01034355	1.82595615	7.10E-18	Transcriptional regulator of the myo-inositol catabolic operon IolR
G200_RS01034400	2.322930823	6.16E-29	5-deoxy-glucuronate isomerase IolB
G200_RS01034455	2.343048715	1.92E-08	Methylmalonate-semialdehyde dehydrogenase IolA
G200_RS01034500	2.393433177	3.58E-08	Inosose isomerase IolI
G200_RS01034855	2.265947451	7.57E-22	Inosose dehydratase
G200_RS01034900	1.606575527	5.42E-13	Myo-inositol 2-dehydrogenase
G200_RS01099455	2.054104613	1.56E-06	Myo-inositol 2-dehydrogenase
G200_RS01117355	2.826752946	1.30E-21	Major myo-inositol transporter IolT
G200_RS01199355	2.36064358	6.17E-31	Inositol transport system permease protein
G200_RS01199400	2.922693363	5.94E-43	Inositol transport system ATP-binding protein
G200_RS01199455	2.723773939	6.09E-34	Inositol transport system sugar-binding protein
G200_RS01200455	2.507147476	1.44E-09	Myo-inositol 2-dehydrogenase 2
D-galactarate, D-glucarate and D-glycerate catabolism			
G200_RS01143555	-1.471923639	1.25E-06	MFS transporter
G200_RS01242800	-2.146858379	7.57E-39	D-galactarate dehydratase GarD
G200_RS01242900	-2.155286292	3.22E-65	D-glucarate permease
G200_RS01242955	-1.762097381	2.90E-22	Glucarate dehydratase GudD
G200_RS01243000	-1.801096855	3.95E-16	Glucarate dehydratase GudD
G200_RS01243055	-1.841655614	7.57E-39	2-dehydro-3-deoxyglucarate aldolase GarL
G200_RS01243200	-1.921850417	2.06E-49	Glycerate kinase
G200_RS25820	-2.073060541	9.97E-53	3-hydroxyisobutyrate dehydrogenase GarR
Maltose and Maltodextrin catabolism			
G200_RS01055200	-1.474187006	1.51E-22	PTS system, maltose and glucose-specific IIABC component
G200_RS01144555	-2.146215061	6.10E-08	Maltose/maltodextrin high-affinity receptor LamB
G200_RS01144600	-3.460976388	1.17E-46	Maltose/maltodextrin transport ATP-binding protein MalK
G200_RS01144655	-3.432632153	3.66E-74	Maltose/maltodextrin ABC transporter, substrate binding periplasmic protein MalE
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584

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